

## Development and evaluation of TUMS medium, a novel biphasic culture medium for isolation of *Brucella* spp. from patients

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### ABSTRACT

**Background and objectives:** There are limitations in time and technique for isolation of *Brucella* from patients. We developed a new *Brucella* culture medium and evaluated its efficiency compared to BACTEC blood culture system and serology.

**Materials and Methods:** A bi-phasic medium containing Urea agar and Brain Heart Infusion was formulated. Appearance of clear red color in liquid phase was the basis of positivity for *Brucella*. The new medium which is designated as TUMS medium (TUMS refers to Tehran University of Medical Sciences) and BACTEC blood culture vials were inoculated with different concentrations of 20 *Brucella* strains. The blood samples from 58 suspected patients were tested by both media and serology (Wright and Coombs). Any growth was sub-cultured and suspected colonies were identified by standard methods.

**Results:** The TUMS medium detected more positive samples (100%) than BACTEC (85%) when the organism was suspended at lower concentration (10 CFU). Of 58 blood cultures, 47 (81%) samples tested on TUMS medium (incubation period =4.2 days) and 39 (67.2 %) samples tested on BACTEC (incubation period =3.3 days) were found positive.

**Conclusion:** The TUMS medium was superior to others in detecting the organism from patients with clinical signs or who took medications for >1year. The TUMS medium is easy to prepare and use in endemic areas where resources are limited.

**Keywords:** *Brucella*, TUMS medium, Blood culture, BACTEC, Iran.

### INTRODUCTION

Brucellosis is endemic in rural areas of Iran and other Middle Eastern countries (1). Diagnosis depends on clinical signs, serological tests, isolation of the organism from blood and other organs by bacterial culture. The definite diagnosis of brucellosis is based on isolation of the bacteria from the patient. However, the available bacterial culture methods are inadequate for screening due to low concentration of the bacteria (1.5 CFU/ml) in the blood of patients (2), long generation time of organism (2.5-3.5 hours) and its intracellular growth (3,4). Other reasons are lack of turbidity

in the liquid culture media and lack of appropriate blood culture media for long incubation periods. Culture in a biphasic medium (Castaneda technique) and lysis concentration have been recommended to improve the recovery of *Brucella* spp. from clinical specimens (5). However, development of colonies may take as long as 35 days even under these conditions. Recently, a new automated system for detection of microbial growth in blood cultures (BACTEC) has been introduced, and it has been reported to detect the organisms from cases with bacteremia and fungaemia in shorter time compared with other currently available blood culture systems (6). Automated blood culture systems allow early detection of microorganisms with continuous monitoring, as well as decreases in the risk of contamination (7). However, the advantages of these systems over conventional culture systems is debatable in cases of slow-growing bacteria such

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as *Brucella* spp. Release of CO<sub>2</sub> by *B. melitensis* is not high enough to be detected as positive by the system and this is clearly preceded by *Brucella* growth.

We developed a new biphasic medium and evaluated its sensitivity for *in vitro* detection of this organism. The new designed medium was named TUMS (Tehran University of Medical Sciences) medium, and used for isolation of *Brucella* from suspected cases. BACTEC Blood Culture System and serology were used in parallel to compare the results.

## MATERIALS AND METHODS

**Preparation of the new medium.** The TUMS medium in this study was a biphasic medium, containing brain and heart infusion (BHI) and urea agar slant with 0.025% sodium polyanethol sulfonate. To formulate this medium, urea agar base (Oxoid, UK) was prepared and autoclaved separately and mixed with the filter sterilized urea powder before it was aseptically poured (30ml) into the 150ml glass bottles. The bottles were placed flat side down on a laboratory bench and left overnight to allow the agar to harden. Subsequently, the autoclaved brain heart infusion broth (Oxoid, UK) was added aseptically to each bottle (50 ml) after cooling. The bottles were all incubated for 48 h. at 35°C to check their sterility. Unlike most commercially available blood culture bottles, no CO<sub>2</sub> or other gaseous mixture was added to the biphasic bottle. The bottles were not treated with vacuum either. In this biphasic medium, solid phase appeared milky in color and broth phase was light brown (Fig. 1.). Although broth phase was BHI broth, some components from the agar phase diffused into the broth phase, making the broth phase components different from BHI broth. Important components in biphasic medium consist of urea in solid phase (2.0 %), urea in liquid phase (0.3%), glucose in liquid phase (0.2%), NaCl (0.5 %), peptone (1.0%) and phenol red. The rate of urea leak from solid phase to liquid phase was 15%.

***In vitro* evaluation of TUMS medium for isolation of *B. melitensis*.** Twenty isolates of *Brucella* spp cultured from patients at Tehran University hospitals were inoculated onto chocolate agar and incubated for 48 h. at 36°C in 5% CO<sub>2</sub>. The

bacterial growth was then suspended in 5 ml of tryptic soy broth (TSB) and their turbidities were adjusted to approximately 0.5 McFarland. This suspension (10<sup>8</sup> CFU/ml) was then diluted serially in TSB to prepare 10<sup>3</sup>, 10<sup>2</sup>, 10 and 1 CFU/ml. The concentrations of bacterial cells were checked by sub-culturing on plates containing chocolate agar and counting colonies. Due to the low number of bacteria present in patients with *Brucella* bacteremia, the inoculate consisted of 10<sup>3</sup>, 10<sup>2</sup>, 10 and 1 CFU/ml in each bottle. The TUMS medium and standard aerobic/F bottles (Becton Dickenson, USA) (Eighty bottles from each) were inoculated with 1 ml of each dilution described above. The TUMS media were incubated at 37°C in a vertical position. They were examined macroscopically for a pink color change every day and the results were recorded. Standard aerobic/F bottles were incubated in BACTEC 9120 system (Becton Dickenson, USA), where they were continuously agitated and monitored. Time was recorded for each of the bottles when the culture became positive. Gram stain and subculture were performed on all TUMS medium and standard aerobic/F bottles when they became positive.

To investigate the effect of urease positive bacteria (i.e., *Proteus mirabilis*, *Morganella morganii*, *Klebsiella oxytoca* and *Oligella ureolytica*) on the TUMS medium, 1ml of each bacterial suspension was inoculated into the TUMS medium after their concentration was adjusted to 0.5 McFarland in tryptic soy broth (TSB).

**Detection of *Brucella* from blood of suspected cases.** Fifty-eight suspected brucellosis patients, 38 (65.5%) males and 20 (34.5%) females, were included in this study. The patients were divided into three groups: group I, individuals with clinical signs and a positive Wright test (= 1/320); group II, individuals with clinical signs and symptoms and a history of direct contact with infected animals or ingestion of un-pasteurized dairy products; group III, individuals infected with *Brucella* who had received appropriate treatment for 12 months but still had ambiguous clinical signs. Similar grouping has been used by Irmak et al. (8) to cluster the patients with brucellosis (8).

Five ml of blood from every patient was inoculated onto the TUMS medium and standard aerobic/F bottles and 2 ml of blood was collected in a tube for serological tests.

**Table 1.** Results of blood culture obtained with BACTEC and the TUMS medium in comparison with serum agglutination test on blood samples from 58 patients.

	Sex			BACTEC			Design medium			SAT	
	Male	Female	Sum	Pos	Mean time	Neg	Pos	Mean time	Neg	Pos	Neg
Group I	19	9	28	25	3	3	28	4		24	4
Group II	7	5	12	12	3.5		12	3		12	
Group III	12	6	18	2	6	16	7	7	11	14	4
Total	38	20	58	39		19	47		11	50	8
Percent			100	67.2		32.8	81		19	86.2	13.8

The TUMS medium was incubated at 37°C in a vertical position and standard aerobic/F bottle was inserted into the BACTEC 9120 system for seven days. Bottles giving a positive growth index and negative bottles were tested by Gram staining and sub-cultured to chocolate agar at 37°C in CO<sub>2</sub>. Subculture and Gram staining were performed on TUMS media every day until they changed color to pink. On macroscopically negative TUMS media, Gram staining and subculture were performed every day for one week and then every week for 5 weeks. Chocolate agar plates were incubated at 37°C in CO<sub>2</sub> for up to 7 days. The Gram-negative coccus-bacilli were identified by conventional biochemical tests (e.g., motility, oxidase, catalase, glucose fermentation and production of H<sub>2</sub>S) and also by an agglutination test using antisera (9).

**Serological tests.** The Rose Bengal (RB) test and the serum agglutination test (SAT) were performed according to standard procedures. Briefly, for the RB test, undiluted serum samples (30 µL) were mixed with an equal volume of Rose Bengal Slide Screening Test antigen (Biotech Laboratories, Barcelona, Spain) on a white agglutination card. Results were rated as negative when agglutination was absent and 1+ to 4+ (positive) on the basis of agglutination strength. The SAT was performed by preparing two-fold serial dilutions of the serum sample starting at a dilution of 1:20 in the wells of a microtiter plate and the addition of an equal volume of stained *Brucella abortus* antigen MM101 (Linear Chemicals, Barcelona, Spain). The mixtures were incubated for 24 hours at 37°C and read visually (8). As the patients lived in rural areas with endemic prevalence of brucellosis, high titer was defined as a titer

of =1/320 in SAT (10). When a SAT result was negative, the test was repeated in high dilution (up to 1/2560) and with Coombs sera to detect non-agglutinating antibodies (11).

## RESULTS

From the 80 bacterial suspensions of 20 isolates of *Brucella*, the TUMS medium and BACTEC 9120 system detected 78 (97.5%) and 72 (90%) respectively. All bacterial suspensions with concentration of 10<sup>3</sup> and 10<sup>2</sup> CFU/ml were detected by the TUMS medium and BACTEC 9120 system. Twenty suspensions (100%) with concentration of 10 CFU/ml of *Brucella* were detected by TUMS medium, while 17 (85%) were detected by BACTEC. Difference between both media was observed in detection of this organism at lower concentration: the TUMS medium and BACTEC detected 18 (90%) and 14 (70%) of the suspensions with 1 CFU/ml respectively.

The distributions of positivity for RB test and SAT for different groups of patients were as follows: group I- 24 (85.7%), group II- 12 (100%) and group III- 14 (77.8%). In total, of the 58 suspected samples, 50 (86.2%) were positive in RB and SAT tests (Table 1). The TUMS medium detected more cases among patients in groups I and III than BACTEC system. The results of both medium for patients in group II were similar.

Of the 58 blood cultures, 47 (81%) were found as positive with TUMS medium (mean detection time, 4.2 days) while 39 (67.2%) became positive using BACTEC 9120 system (mean detection time, 3.3 days). Eleven blood cultures were negative by both media after 5 weeks (Table 1). Thus BACTEC 9120 system had 13.8% false negative results. All the bacterial isolates were identified as *B. melitensis*.

## DISCUSSION

By designing the new biphasic TUMS culture medium, we improved the sensitivity and cost of culture for isolation of *Brucella*. To achieve this, we used production of urease by the organism. Urea agar and BHI broth were chosen as the solid phase and the liquid phase respectively. BHI does not contain any indicator. However, phenol red can diffuse from the solid phase into the liquid phase. The final pH of the liquid phase is 7.2. Phenol red turns yellow at pH 6.8 (acidic) and red-pink at pH 8.4 (alkaline). Therefore, in this culture medium, a change in color of culture medium can be used for detection of *Brucella* growth, which is much simpler than fluorescent based technology such as BACTEC. Urea in liquid phase causes a more rapid color change. This change occurs 12-14 hours earlier than the time when the solid phase becomes red-pink. Therefore, there is no need for several subcultures for diagnosis of brucellosis and identification of the organism involved.

Glucose is another important compound of this new culture medium. It is available only in liquid phase (0.2g %). The glucose present in the liquid phase removes the possible false negative results in bacteria that hydrolyze urea slowly and increases their urease activity. The energy released from glucose increases the metabolism and the bacterial multiplication and eventually the bacterial urease production and activity.

Glucose not only accelerates detecting urea positive bacteria but also accelerates detection of urea negative bacteria that are not able to hydrolyze urea as the medium turns acidic and quite yellow due to fermentation.

The clarity of the culture medium after growth is an important factor for interpretation of results. In ordinary blood culture media and in even the Castaneda blood culture medium, the clarity can mislead laboratory staff to report negative results. The clarity of our designed medium is one of its advantages since the presence of *Brucella* changes the color of the culture media (Fig. 1). It gives evidence to differentiate *Brucella* from several other urease positive bacteria such as *Proteus* and *Klebsiella*. Therefore, in a clear medium, any changes in color can be reported as positive without any requirement for sub-culture and further examination.

In this study, the number of positive cases obtained from the TUMS culture medium was higher than that being obtained from BACTEC that shows

higher instances of false negative cases in BACTEC. Consequently, it is necessary to obtain subcultures from the negative culture vials, particularly for bacteria with slow metabolism and growth.

The mean time for detection of *Brucella* was shorter in BACTEC. However, the problem of false negative results with BACTEC is considerable (12,13). The difference between the numbers of cases detected by BACTEC with respect to those detected by this newly designed medium amongst the patients in group III, i.e. those being treated for 1- 4 years was considerable. In two patients of this group the results obtained with both blood culture media were positive. However, in five other patients within this group, the results of BACTEC blood culture were negative, but the TUMS medium identified these to be positive. Since patients in this group had already taken anti-brucellosis drugs, it appears that the organism guaranteed its viability by lowering its metabolism. This can lead to reduction in CO<sub>2</sub> production and eventually prevent detection by BACTEC.



**Fig. 1.** The TUMS medium before inoculation with *Brucella* (pre - culture). The solid phase appears as milky in color and the broth phase looks as light brown (post - culture).



Concerning the 11 patients whose blood culture yielded negative results, the bacteria might have localized to their tissues or changed to the L form (14). All had disease symptoms and were positive in Wright test. Thirty-nine patients were found positive with both BACTEC and TUMS medium. The main advantages of TUMS medium are as follows: (1) lower cost, (2) availability of the components in the small labs and rural areas, (3) simplicity and decisiveness in interpretation due to clear color change, (4) greater safety as it does not require additional sub-culture and (5) higher sensitivity in comparison with BACTEC.

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